Circular RNAs in urine of kidney transplant patients with acute T Cell-mediated allograft rejection

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Abstract: BACKGROUND: Circular RNAs (circRNAs) have recently been described as novel noncoding regulators of gene expression. They are detectable in the blood of patients with acute kidney injury. We tested whether circRNAs were present in urine and could serve as new predictors of outcome in renal transplant patients with acute rejection. METHODS: A global circRNA expression analysis using RNA from urine of patients with acute T cell-mediated renal allograft rejection and control transplant patients was performed. Dysregulated circRNAs were confirmed in a cohort of 62 patients with acute rejection, 10 patients after successful antirejection therapy, 18 control transplant patients without rejection, and 13 stable transplant patients with urinary tract infection. RESULTS: A global screen revealed several circRNAs to be altered in urine of patients with acute rejection. Concentrations of 2 circRNAs including hsa_circ$_0$001334*andhsa_circ*$_0$71475*weresignificantlyincreased.Thesewerevalidatedinthewholecohortofpatients.hsa_CircRNAconcentrationsaresignificantlydysregulatedinpatientswithacuterejectionatsubclinicaltimepoints.Urinaryh*

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Circular RNAs in Urine of Kidney Transplant Patients with Acute T Cell-Mediated Allograft Rejection

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BACKGROUND: Circular RNAs (circRNAs) have recently been described as novel noncoding regulators of gene expression. They are detectable in the blood of patients with acute kidney injury. We tested whether circRNAs were present in urine and could serve as new predictors of outcome in renal transplant patients with acute rejection.

METHODS: A global circRNA expression analysis using RNA from urine of patients with acute T cell-mediated renal allograft rejection and control transplant patients was performed. Dysregulated circRNAs were confirmed in a cohort of 62 patients with acute rejection, 10 patients after successful antirejection therapy, 18 control transplant patients without rejection, and 13 stable transplant patients with urinary tract infection.

RESULTS: A global screen revealed several circRNAs to be altered in urine of patients with acute rejection. Concentrations of 2 circRNAs including *hsa_circ_0001334* and *hsa_circ_0071475* were significantly increased. These were validated in the whole cohort of patients. *hsa_circ_0001334* was upregulated in patients with acute rejection compared with controls. Concentrations of *hsa_circ_0001334* normalized in patients with acute rejection following successful antirejection therapy. *hsa_circ_0001334* was associated with higher decline in glomerular filtration rate 1 year after transplantation.

CONCLUSIONS: CircRNA concentrations are significantly dysregulated in patients with acute rejection at subclinical time points. Urinary *hsa_circ_0001334* is a novel biomarker of acute kidney rejection, identifying patients with acute rejection and predicting loss of kidney function.

Chronic allograft dysfunction is a major contributor to impairment of long-term kidney function, graft loss, and survival of patients *(1)*. It is itself a consequence of acute rejection episodes of renal allografts *(1)*. The use of potent immunosuppressants has steeply reduced the incidence of acute rejection *(2)*. Timely detection of acute rejections in patients after kidney transplantation is of utmost importance to initiate an immunosuppressive antirejection therapy without delay, thereby preventing severe injury of the graft. Histological assessment of kidney biopsy specimens is still necessary and most precise in the diagnosis of acute rejection episodes. It also enables identification of subclinical rejection, which is defined by histological lesions without a change in kidney function parameters, e.g., serum creatinine *(3, 4)*. The invasive nature of kidney biopsies and their associated potentially severely harmful side effects remain a challenge as far as patient safety is concerned. Kidney function may be assessed noninvasively by the use of creatinine in blood samples. However, creatinine assessment is sometimes unreliable in precisely identifying a patient's kidney function because of its dependency on muscle mass. Moreover, increases in creatinine concentrations occur with a time lag and at later stages with already progressed kidney damage and, therefore, are not sufficient to detect early rejection *(5)*. Thus, novel biomarkers, combining clinical sensitivity, specificity, and noninvasiveness, are desired. A large part of the human genome (>90%) is transcribed into RNA transcripts without protein-coding potential *(6)*. Based on their size, these so-called noncoding RNAs (ncRNAs)[4] are arbitrarily separated into long ncRNAs (lncRNAs, 200 nucleotides) and small ncRNAs (200 nucleotides). MicroRNAs (miRNAs) and lncRNAs have also been investigated *(7–10)*. miRNA activity has been shown to be affected by the presence of miRNA sponge transcripts, the so-called competing endogenous RNA. Circular RNAs (circRNAs), which are themselves considered lncRNAs, are part of the aforementioned competing endogenous RNA. They are endogenously expressed as single-stranded, covalently closed circular mol-

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[4] Nonstandard abbreviations: ncRNA, noncoding RNA; lncRNA, long noncoding RNA; miRNA, microRNA; circRNA, circular RNA; GFR, glomerular filtration rate.

ecules *(11)*. CircRNAs are secreted into the bloodstream with a remarkable stability owing to resistance to exonucleases through circularization. We recently performed a study investigating the presence of circRNAs in blood of patients with acute kidney injury *(12)*. However, because of its accessibility, urine is the ideal specimen to detect intrarenal changes. Therefore, we investigated here the predictive nature of circRNAs in urine of renal transplant patients with acute T cell-mediated rejection concerning acute rejection (primary outcome measure) and loss of glomerular filtration rate at 1 year after transplantation (secondary outcome measure).

Materials and Methods

PATIENTS AND SAMPLE COLLECTION

The study was approved by the Ethical Committee of the Hanover Medical School. All patients received and signed written informed consent. At our transplant center, renal protocol biopsies are regularly performed after 6 weeks, 3 and 6 months following kidney or combined kidney/pancreas transplantation. Midstream spot-urine samples were collected immediately before biopsy collection and subsequently frozen at −80 °C. Routine analyses of fresh urine samples include determination of protein concentration and screening for hematuria and leukocyturia by dipstick analysis and microscopic inspection. The available urine samples were divided into 2 groups according to the files from patients who participated in the protocol biopsy program: (*a*) transplant patients with biopsy-proven acute cellular rejection (n = 62) and (*b*) transplant patients with stable kidney function without evidence of acute rejection (n = 31) as controls. Fifty-one patients presented with subclinical rejection, which was defined as a biopsy-proven rejection without changes in serum creatinine. Eleven patients had a clinically detectable rejection (change in serum creatinine by >20%). In total, acute cellular rejection could be detected in 20 urine samples from patients at 6 weeks, 28 urine samples at 3 months, and 14 urine samples at 6 months after kidney transplantation. Forty-four patients presented with *Ban*ff IA rejection, 9 with *Ban*ff IB, 8 with *Ban*ff 2A, and 1 patient with *Ban*ff 2B rejection. In addition, 10 urine samples after successful antirejection therapy of patients with acute rejection were also analyzed. Interpretation of biopsies was performed according to the updated *Ban*ff 2009 classification *(13)*. Glomerular filtration rate (GFR) was calculated according to the Cockcroft–Gault formula. All samples were processed within 4 h.

A detailed description of the study outcomes and statistical analysis, RNA isolation, circRNA transcriptome analysis, and circRNA validation are provided in the Materials and Methods file found in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol65/issue10. The most highly dysregulated circRNAs are shown in Table 1 of the online Data Supplement.

Results

circRNA EXPRESSION ANALYSIS IN URINE

To test the predictive value of urinary circRNAs in renal transplant patients with acute T cell-mediated rejection, we performed a genome-wide circRNA expression analysis in urine of patients with T cell-mediated rejection (n = 9) and age-matched control transplant patients with stable function (n = 9). In total, 5119 circRNAs were detected and 363 circRNAs with signal intensities >4 were differentially expressed (see Table 1 in the online Data Supplement). Fig. 1 shows a heatmap of the most strongly differentially expressed and subsequently hierarchically clustered circRNAs. A distinct expression signature of circRNAs could clearly detect rejection patients. The variation in circRNA expression is displayed as a scatter plot in Fig. 2A. Fold changes in relation to *P* values were visualized as a volcano plot in Fig. 2B. To identify promising circRNA candidates for subsequent biomarker analysis, we focused on candidates with high signal intensities in all groups to guarantee stable detection but also on high differential expression. CircRNA candidates *hsa_circ_0071475* and *hsa_circ_0001334* were selected for further validation studies in the whole cohort (marked in Fig. 2, A and B). *hsa_circ_0071475* was chosen specifically because of its high fold change, and *hsa_circ_0001334* was selected because of its additional high signal intensity in all groups. An overview of the 20 most strongly upregulated circRNAs with signal intensities >6 is summarized in Table 2 of the online Data Supplement, and the top 20 downregulated circRNAs with a signal intensity >6 are displayed in Table 3 of the online Data Supplement.

circRNA VALIDATION IN URINE

To investigate the detectability of dysregulated urinary circRNAs, we assessed the 20 most highly dysregulated circRNAs using RNA isolated from urine of patients with transplant rejection in agarose gel electrophoresis. Here, we found 2 circRNAs to display specific bands of correct size, including *hsa_circ_0071475* and *hsa_circ_0001334*. We then performed real-time PCR analysis in a subset of patients with acute T cell-mediated rejection (n = 10). These 2 novel transcripts showed clean amplification curves and specific curves in melting curve analysis and were undetectable in water controls without template. To ascertain that transcripts were specifically detected in urine, we sequenced transcripts in these patients after PCR amplification, which confirmed that circRNAs were correctly amplified and detectable.

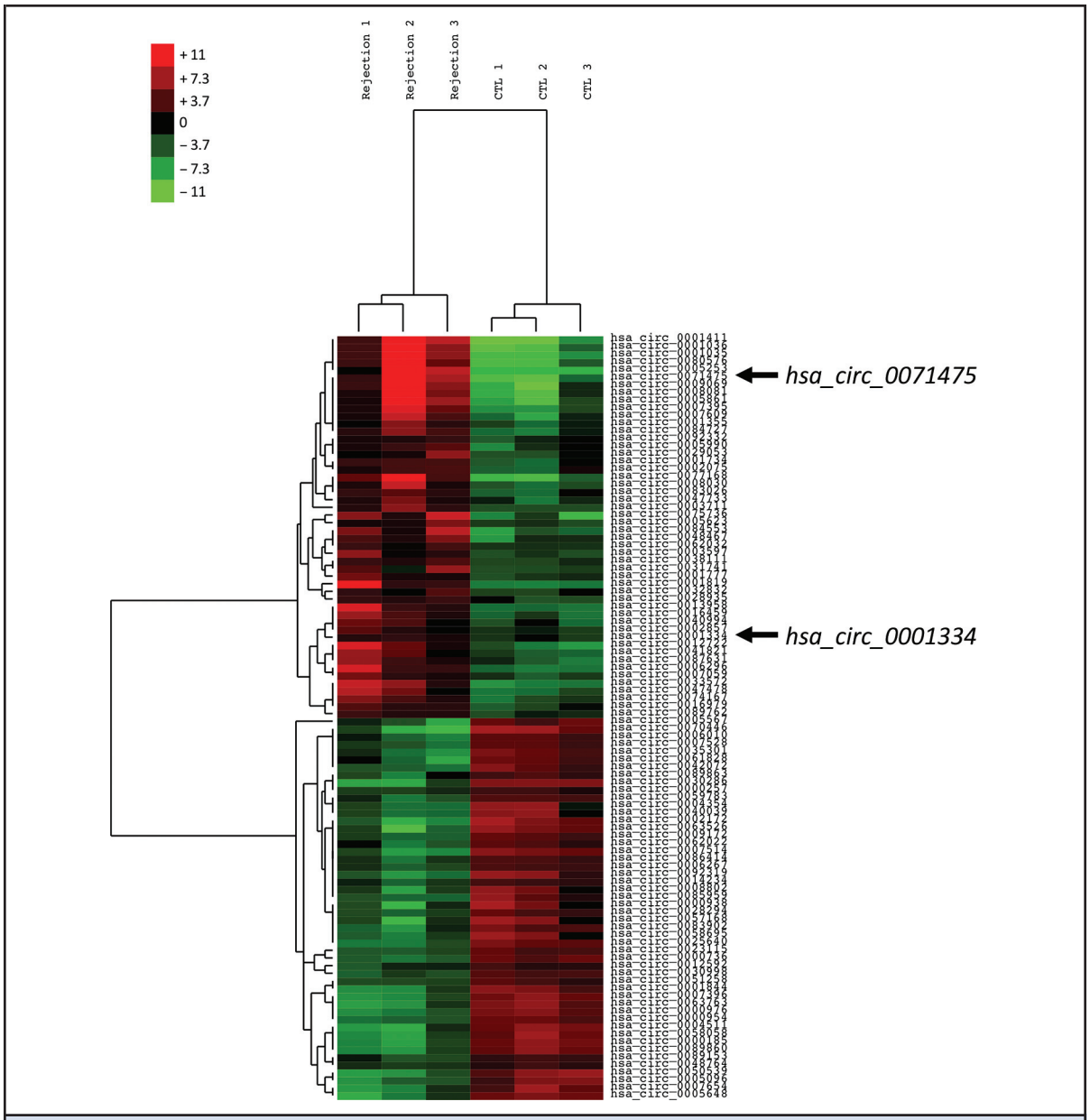

Fig. 1. Whole-genome circRNA expression analysis: hierarchical cluster of dysregulated circRNAs in urine from patients with T cell-mediated acute renal allograft rejection vs control transplant patients with stable kidney function.

Downregulated circRNAs are depicted in green and upregulated in red; 2 investigated circRNAs in the whole cohort are marked. Each column represents pooled RNA from 3 patients.

Primer sequences of tested circRNAs are given in Table 4 of the online Data Supplement.

hsa_circ_0001334 IS A NOVEL BIOMARKER OF ACUTE T CELL-MEDIATED REJECTION

We next compared the concentrations of identified transcripts *hsa_circ_0071475* and *hsa_circ_0001334* between 18 samples from stable controls without rejection, 13 transplant patients with urinary tract infection (disease controls), 62 samples of patients with acute T cell-mediated rejection, and 10 samples from patients following successful antirejection therapy (these samples were chosen from the 62 samples with acute rejection at a different time point after transplantation). As shown in Fig. 3A, *hsa_circ_0071475* could not be confirmed to be differentially regulated in patients with acute T cell-

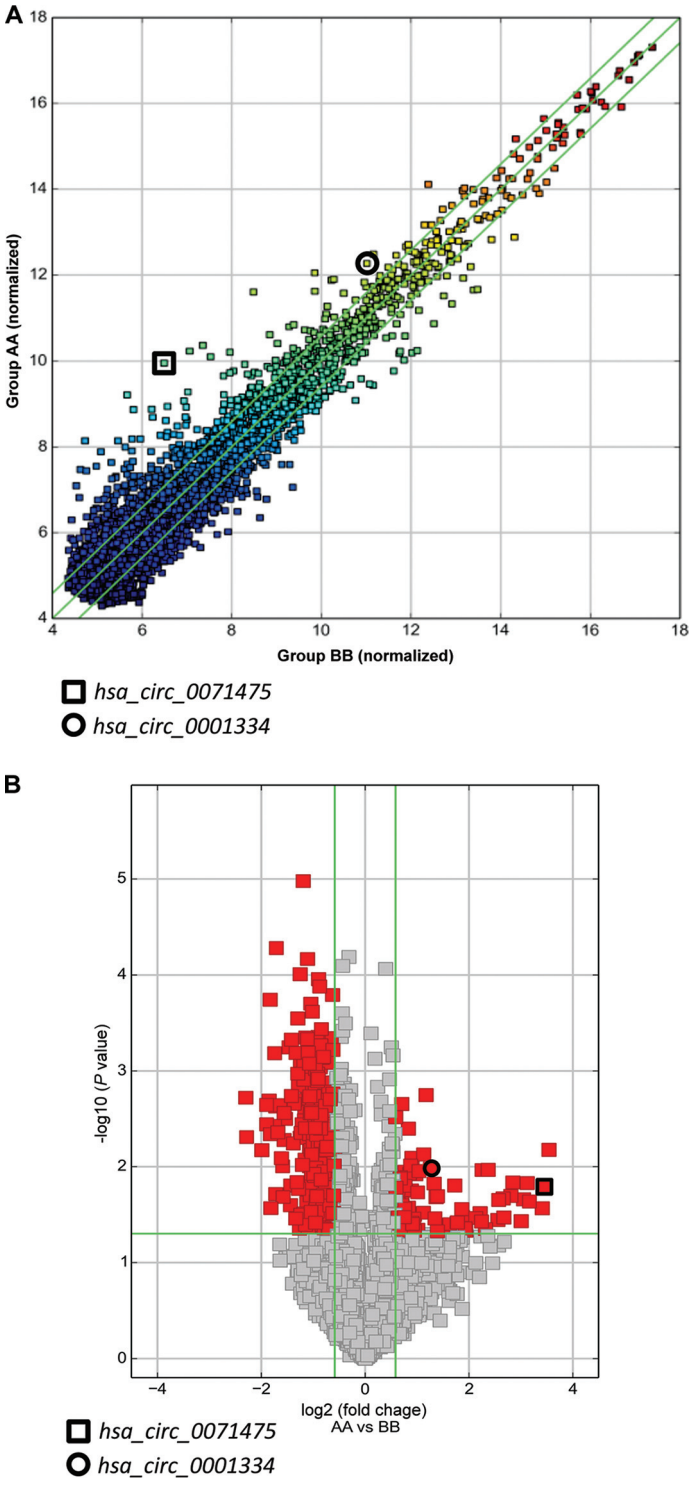

Fig. 2. Scatter plot (A) and volcano plot analysis (B) of dysregulated circRNAs in urine of patients with acute rejection and stable controls.

Identified circRNA are further marked: *hsa_circ_0001334* (black circle) and *hsa_circ_0071475* (black square). Gray color represents nonsignificantly altered transcripts; red color represents significantly altered transcripts.

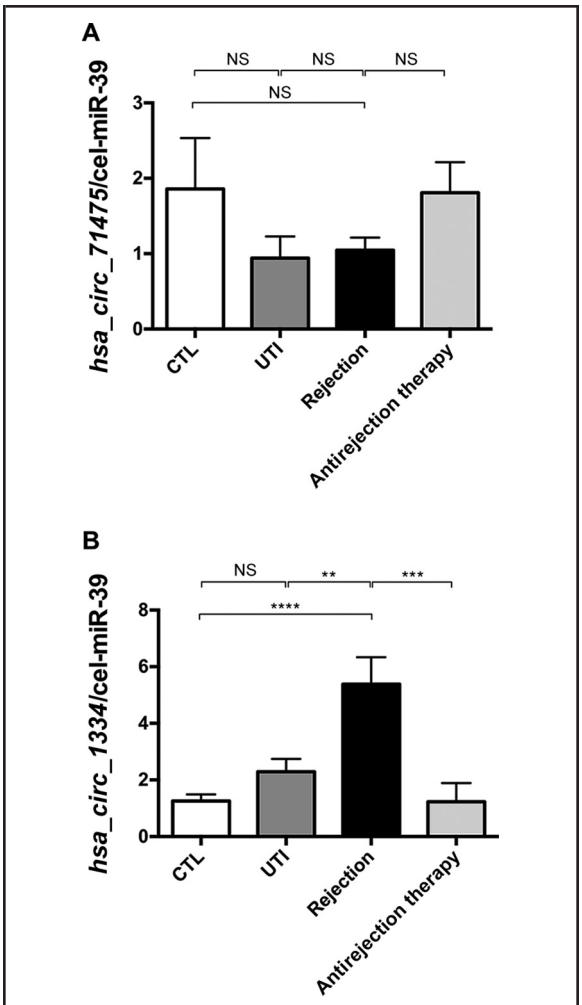

Fig. 3. Validation of urinary circRNA concentrations in the whole cohort of patients.

(A), Concentrations of *hsa_circ_0071475* in patients with acute T cell-mediated rejection compared with control patients (CTL) with stable transplant function and transplant patients with urinary tract infection (UTI) as well as in patients after successful antirejection therapy. (B), Concentrations of *hsa_circ_0001334* in patients with acute T cell-mediated rejection compared with control patients with stable transplant function and transplant patients with UTI as well as in patients after successful antirejection therapy. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

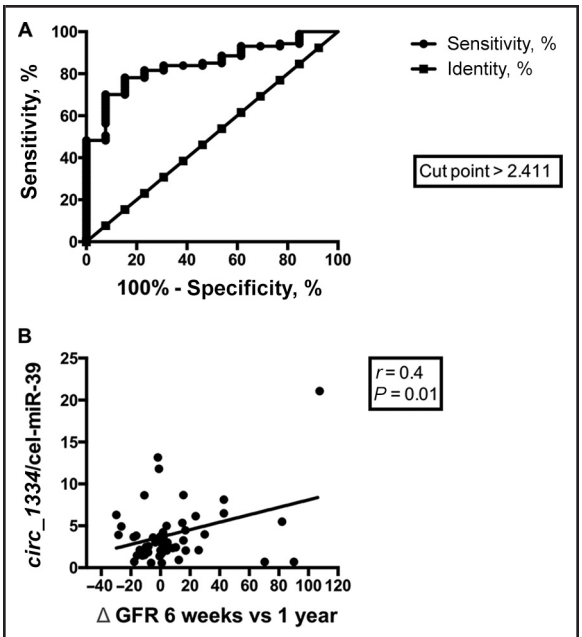

Fig. 4. Sensitivity, specificity, and predictive value of *hsa_circ_0001334*.

(A), ROC curve analysis of patients with acute T cell-mediated rejection compared with stable control patients regarding urinary *hsa_circ_0001334*. Urinary *hsa_circ_0001334* predicts long-term outcome of renal transplant patients with acute rejection. (B), High *hsa_circ_0001334* concentrations ($r = 0.4$; $P = 0.01$) were significantly associated with decline in GFR 1 year after transplantation.

mediated rejection. The concentrations of the second candidate *hsa_circ_0001334* were significantly increased ($P < 0.0001$) in patients with acute T cell-mediated rejection compared with stable transplant controls without signs of rejection (Fig. 3B). In addition, it was not significantly different in kidney transplant patients with urinary tract infection, indicating its clinical specificity as a biomarker of acute rejection episodes (Fig. 3B). Moreover, *hsa_circ_0001334* concentrations normalized in 10 patients with acute T cell-mediated rejection after successful antirejection therapy (Fig. 3B here and also Fig. 1 in the online Data Supplement). To assess the biomarker performance of *hsa_circ_0001334*, we performed an ROC curve analysis (Fig. 4A), which indicated an area under the curve of 0.85 ($P < 0.0001$). A cut point of 2.41 was associated with a clinical sensitivity of 70.11% (95% CI, 59.35%–79.46%) and a clinical specificity of 92.31% (95% CI, 63.97%–99.81%) and a likelihood ratio of 9.115. The positive predictive value of this cut point was calculated to be 98.39%, and the negative predictive value was 31.58%. Moreover, *hsa_circ_0001334* ($r = 0.4$; $P = 0.01$) was significantly associated with decline in GFR 6 weeks after transplantation compared with 1 year after transplantation (Fig. 4B), supporting a higher GFR loss in patients with high concentrations of urinary *hsa_circ_0001334*. Fig. 2A in the online Data Supplement shows the changes in serum creatinine concentrations before and at the time of rejection. Fifty-one patients presented with subclinical rejection (no changes in serum creatinine), and 11 patients presented with clin-

ically detectable rejection (changes in serum creatinine). *hsa_circ_0001334* detects patients with subclinical rejection, which would have been missed by routine serum creatinine measurements (see Fig. 2B in the online Data Supplement). Therefore, we propose *hsa_circ_0001334* as a marker of acute rejection, which is superior to measuring serum creatinine changes in patients.

hsa_circ_0001334 SPONGES *hsa-miR-4459, hsa-miR-665, hsa-miR-6514-3p, hsa-miR-4739,* AND *hsa-miR-6893-5p*

CircRNAs are involved in diverse biological functions. One of the most important functions is characterized by sponging and sequestering miRNAs *(11)*. Therefore, we tested whether *hsa_circ_0001334* contained binding sites for miRNAs. We used the bioinformatic TargetScan and miRanda algorithms and identified binding sites for 5 different miRNAs: *hsa-miR-4459*, *hsa-miR-665*, *hsa-miR-6514-3p, hsa-miR-4739*, and *hsa-miR-6893-5p* (see Fig. 3 in the online Data Supplement). More specifically, *hsa_circ_0001334* contained 8 binding sites for *hsa-miR-4459* and 6 binding sites for *hsa-miR-665*, *hsa-miR-6514-3p*, *hsa-miR-4739*, and *hsa-miR-6893-5p*, respectively (see Fig. 3, A-E, in the online Data Supplement).

Discussion

We performed a clinical evaluation of urinary circRNAs in patients with transplant- associated kidney disease. Our results are as follows. First, the detection of circRNAs in urine was feasible. Second, a variety of circRNAs were significantly ($P < 0.05$) dysregulated in urine of transplant patients. Third, in a validation cohort the concentration of transcript *hsa_circ_0001334* was up-regulated in patients with acute rejection compared with controls with stable graft function and disease controls with urinary tract infection. Although the fold change of *hsa_circ_0071475* was larger than the one of our final candidate *hsa_circ_0001334*, the signal intensities of *hsa_circ_0071475* were much lower than the ones of *hsa_circ_0001334* (see Tables 1 and 2 in the online Data Supplement). Consequently, we found that a high signal intensity as shown for *hsa_circ_0001334* was of much higher diagnostic value because differentiation of patients and controls was constantly guaranteed. Fourth, the concentrations of *hsa_circ_0001334* were normalized in patients with acute rejection following successful antirejection therapy. Fifth, the concentrations of *hsa_circ_0001334* were predictive concerning subsequent loss of kidney function.

Our study demonstrates that urinary circRNAs in patients with any type of kidney disease can be stably isolated and detected. As previously shown for miRNAs *(14, 15)* and lncRNAs *(16, 17)*, a stable control or "housekeeping" circRNA has not been defined in body fluids of patients. Therefore, we supplemented recombinant *Caenorhabditis elegans*–miR-39 to urine samples during the RNA isolation process to normalize for potential differences in RNA isolation efficiency and relate concentrations of urinary circRNAs to these levels of known concentration. This approach was previously shown for blood-derived circRNAs *(12)*.

In search of adequate biomarkers of early detection of acute renal allograft rejection, an extensive body of literature has been produced investigating circulating plasma or serum concentrations as well as urinary concentrations of adhesion molecules, cytokines, mRNAs, and lymphocyte expression levels (e.g., perforin, granzyme B, and FAS ligand) *(18)*. However, none of these markers have entered routine clinical practice so far.

More than 90% of the human genome is transcribed into RNA transcripts without protein-coding potential *(10)*. Small RNAs have been the subject of increased research initiatives as regulators of disease and biomarkers of disease initiation and response to therapy *(7–10, 14–17)*. On the contrary, the focus has only recently shifted toward analysis of circRNA dysregulation. Their function and biomarker potential are largely unknown to date. CircRNAs share the potential of gene regulation as previously shown for miRNAs and lncRNAs *(19–21)*. CircRNAs have been described to be abundant in the eukaryotic transcriptome and to pose intricate functions. CircRNAs have a length of approximately 100 nucleotides and are secreted into exosomes *(22)*. CircRNAs are generated through a mechanism known as back-splicing "tail" to "head," whereby an exon at the 3′ end of a gene is back-spliced to an exon at the 5′ end of the gene resulting in a circRNA form *(19, 20)*. They can arise mainly from exons, but circRNAs deriving from intergenic or intragenic and intronic regions as well as antisense sequences have been reported *(19, 20)*. Owing to their circular structure and the absence of a 5 cap, it is currently believed that circRNAs are not translated into protein *(23)*. CircRNAs are believed to have diverse functions, the most important likely being the sponging and sequestering of miRNAs *(22, 24)*. This interaction might only be observed in circRNAs with a high number of binding sites for a specific miRNA, such as ciRs-7 (circular RNA sponge for miR-7), for which an excess of 70 conserved miR-7 target sites has been reported *(24)*. CircRNAs show a highly enhanced half-life as compared with their linear counterparts. This is because of their circular structure, which results in lessened susceptibility to exonucleases *(22)*. This stability may offer an improved suitability as stable biomarkers in body fluids.

We previously identified ciRs-126 (circular RNA sponge of miR-126) as a promising new circRNA biomarker in the diagnosis and prognosis of patients with acute kidney injury *(12)*. We present here a study on the expres-

Table 1. Demographic, clinical, and laboratory characteristics of patients.

	Total	No rejection	Rejection	*P* value
Number of patients	93	31	62	0.2
Male, n (%)	54 (58)	18	36	
Female, n (%)	39 (42)	13	26	
Age, years (minimum–maximum)	51 (18–73)	53 (18–68)	51 (21–73)	0.5
Primary transplant, n	81	25	56	0.2
Additional pancreas Tx[a], n	3	2	1	0.3
Type of allograft, n				0.4
Deceased donor	80	28	52	
Living donor	13	3	10	
Initial graft function, n	72	23	49	0.6
Need for HD post-Tx, n	20	6	14	0.8
HLA mismatch, n				
Locus A (0/1/2)	48/35/10	18/8/5	30/27/5	0.2
Locus B (0/1/2)	40/37/16	17/10/4	23/27/12	0.3
Locus DR (0/1/2)	32/45/16	18/12/1	14/33/15	0.001[b]
Donor CMV status, n (positive/negative)	53/40	11/20	42/20	0.004[b]
Type of RRT before Tx (HD/PD/preemptive)	83/8/2	28/3/0	55/5/2	0.6
Diabetes mellitus				0.5
Type 1	4	2	2	
Type 2	5	1	4	
Hyperparathyroidism before Tx	28	5	23	0.05
Initial immunosuppression, n				
Cyclosporine	73	26	47	0.4
Tacrolimus	17	5	12	0.8
Mycophenolate mofetil	55	19	36	0.8
Azathioprine	1	0	1	0.6
Sirolimus	7	1	6	0.4
Steroids	88	30	58	0.7
Preformed antibodies (>1%)	4	3	1	0.1

[a] Tx, transplant; HD, hemodialysis; CMV, cytomegalovirus; RRT, renal replacement therapy; PD, peritoneal dialysis; HLA, human leukocyte antigen.
[b] $p < 0.01$.

sion of circRNAs in urine of transplant patients. The use of urinary biomarkers is a huge advantage in the care of patients with kidney disease because they can be easily assessed in a timely manner without exposing patients to harmful side effects of the kidney biopsy procedure. Given the high clinical sensitivity and specificity as a biomarker, assessment of urinary concentrations of *hsa_circ_0001334* might be considered as an improvement of patient surveillance following kidney transplantation.

There are several distinct possible sources of circulating circRNAs. In hematopoietic cells including progenitors and differentiated myeloid and lymphoid cells, circRNA expression can be cell-specific and increases during cellular maturation *(25)*. Interestingly, enucleated cells such as red blood cells and platelets appear to express higher concentrations of circRNAs as compared with nucleated hematopoietic cells *(26, 27)*. Platelets, in particular, express the highest number of circRNAs, almost twice as much as red blood cells and 5 times more than granulocytes *(26, 27)*. Small vesicles including exosomes carry large amounts of circRNAs *(28)*. The biomarker potential of circRNAs has recently been demonstrated for a variety of patient cohorts, including atherosclerosis *(29)*, disorders of the central nervous system *(30)*, and cancers *(28, 31)*.

There are important limitations to our study: We do not provide molecular insights into the underlying mechanisms of urinary circRNA release. Our study represents

a single center experience with a limited number of patients. Larger independent cohorts are necessary to validate our findings.

In conclusion, we analyzed the potential of urinary circRNAs as a marker of acute rejection in kidney transplant patients. Urinary circRNAs can be stably detected in urine. We identified *hsa_circ_0001334* as a marker of acute rejection and predictor of subsequent loss of graft function. The fact that *hsa_circ_0001334* increases specifically with the development of acute rejection and normalizes to concentrations of stable controls with successful antirejection therapy provides evidence for *hsa_circ_0001334* as a novel biomarker of acute rejection with the potential for clinical application to monitor rejection episodes without the potential harms associated with an invasive renal allograft biopsy.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

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References

1. Almond PS, Matas A, Gillingham K, Dunn DL, Payne WD, Gores P, et al. Risk factors for chronic rejection in renal allograft recipients. Transplantation 1993;55:752.
2. Hardinger KL, Brennan DC. Novel immunosuppressive agents in kidney transplantation. World J Transplant 2013;3:68–77.
3. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR. Natural history, risk factors, and impact of subclinical rejection in kidney transplantation. Transplantation 2004;78:242–9.
4. Moreso F, Ibernon M, Goma M, Carrera M, Fulladosa X, Hueso M, et al. Subclinical rejection associated with chronic allograft nephropathy in protocol biopsies as a risk factor for late graft loss. Am J Transplant 2006;6:747–52.
5. Sanjeevani S, Pruthi S, Kalra S, Goel A, Kalra OP. Role of neutrophil gelatinase-associated lipocalin for early detection of acute kidney injury. Int J Crit Illn Inj Sci 2014;4:223–8.
8. ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. Nature 2012;489:57–74.
9. Lorenzen JM, Thum T. Circulating and urinary microRNAs in kidney disease. Clin J Am Soc Nephrol 2012;7:1528–33.
10. Lorenzen JM, Thum T. Long noncoding RNAs in kidney and cardiovascular diseases. Nat Rev Nephrol 2016;12:360–73.
11. Jeck WR, Sharpless NE. Detecting and characterizing circular RNAs. Nat Biotechnol 2014;32:453–61.
12. Kölling M, Seeger H, Haddad G, Kistler A, Nowak A, Faulhaber-Walter R, et al. The circular RNA ciRs-126 predicts survival in critically ill patients with acute kidney injury. Kidney Int Rep 2018;3:1144–52.
13. Sis B, Mengel M, Haas M, Colvin RB, Halloran PF, Racusen LC, et al. Banff '09 meeting report: antibody mediated graft deterioration and implementation of Banff working groups. Am J Transplant 2010;10:464–71.
14. Lorenzen JM, Kielstein JT, Hafer C, Gupta SK, Kumpers P, Faulhaber-Walter R, et al. Circulating miR-210 predicts survival in critically ill patients with acute kidney injury. Clin J Am Soc Nephrol 2011;6:1540–6.
15. Lorenzen JM, Volkmann I, Fiedler J, Schmidt M, Scheffner I, Haller H, et al. Urinary miR-210 as a mediator of acute T-cell mediated rejection in renal allograft recipients. Am J Transplant 2011;11:2221–7.
16. Lorenzen JM, Schauerte C, Kielstein JT, Hubner A, Martino F, Fiedler J, et al. Circulating long noncoding RNATapSaki is a predictor of mortality in critically ill patients with acute kidney injury. Clin Chem 2015;61:191–201.
17. Lorenzen JM, Schauerte C, Kolling M, Hubner A, Knapp M, Haller H, Thum T. Long noncoding RNAs in urine are detectable and may enable early detection of acute T cell-mediated rejection of renal allografts. Clin Chem 2015;61:1505–14.
18. Gwinner W. Renal transplant rejection markers. World J Urol 2007;25:445–55.
19. Ashwal-Fluss R, Meyer M, Pamudurti NR, Ivanov A, Bartok O, Hanan M, et al. circRNA biogenesis competes with pre-mRNA splicing. Mol Cell 2014;56:55–66.
20. Starke S, Jost I, Rossbach O, Schneider T, Schreiner S, Hung LH, Bindereif A. Exon circularization requires canonical splice signals. Cell Rep 2015;10:103–11.
21. Memczak S, Papavasileiou P, Peters O, Rajewsky N. Identification and characterization of circular RNAs as a new class of putative biomarkers in human blood. PLoS One 2015;10:e0141214.
22. Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, et al. Circular RNAs are a large class of animal RNAs with regulatory potency. Nature 2013;495:333–8.
23. Li XF, Lytton J. A circularized sodium-calcium exchanger exon 2 transcript. J Biol Chem 1999;274:8153–60.
24. Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J. Natural RNA circles function as efficient microRNA sponges. Nature 2013;495:384–8.
25. Salzman J, Gawad C, Wang PL, Lacayo N, Brown PO. Circular RNAs are the predominant transcript isoform from hundreds of human genes in diverse cell types. PLoS One 2012;7:e30733.
26. Preusser C, Hung LH, Schneider T, Schreiner S, Hardt M, Moebus A, et al. Selective release of circRNAs in platelet-derived extracellular vesicles. J Extracell Vesicles 2018;7:1424473.
27. Alhasan AA, Izuogu OG, Al-Balool HH, Steyn JS, Evans A, Colzani M, et al. Circular RNA enrichment in platelets is a signature of transcriptome degradation. Blood 2016;127:e1–11.
28. Li Y, Zheng Q, Bao C, Li S, Guo W, Zhao J, et al. Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. Cell Res 2015;25:981–4.
29. Burd CE, Jeck WR, Liu Y, Sanoff HK, Wang Z, Sharpless NE. Expression of linear and novel circular forms of an INK4/ARF-associated non-coding RNA correlates with atherosclerosis risk. PLoS Genet 2010;6:e1001233.
30. Lukiw WJ. Circular RNA (circRNA) in Alzheimer's disease (AD). Front Genet 2013;4:307.
31. Li P, Chen S, Chen H, Mo X, Li T, Shao Y, et al. Using circular RNA as a novel type of biomarker in the screening of gastric cancer. Clin Chim Acta 2015;444:132–6.